

# The Conversion of Active to Latent Plasminogen Activator Inhibitor-1 Is an Energetically Silent Event

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**ABSTRACT** PAI-1 is a proteinase inhibitor, which plays a key role in the regulation of fibrinolysis. It belongs to the serpins, a family of proteins that behave either as proteinase inhibitors or proteinase substrates, both reactions involving limited proteolysis of the reactive center loop and insertion of part of this loop into  $\beta$ -sheet A. Titration calorimetry shows that the inhibition of tissue-type plasminogen and pancreatic trypsin are exothermic reactions with  $\Delta H = -20.3$ , and  $-22.5$  kcal.mol<sup>-1</sup>, respectively. The *Pseudomonas aeruginosa* elastase-catalyzed reactive center loop cleavage and inactivation of the inhibitor is also exothermic ( $\Delta H = -38.9$  kcal.mol<sup>-1</sup>). The bacterial elastase also hydrolyses peptide-bound PAI-1 in which acetyl-TVASSSTA, the octapeptide corresponding to the P<sub>14</sub>-P<sub>7</sub> sequence of the reactive center loop is inserted into  $\beta$ -sheet A of the serpin with  $\Delta H = -4.0$  kcal.mol<sup>-1</sup>. In contrast,  $\Delta H = 0$  for the spontaneous conversion of the metastable active PAI-1 molecule into its thermodynamically stable inactive (latent) conformer although this conversion also involves loop/sheet insertion. We conclude that the active to latent transition of PAI-1 is an entirely entropy-driven phenomenon.

## INTRODUCTION

The inhibition of proteinases by protein proteinase inhibitors is an individual case of protein-protein interaction. Two major types of inhibitors regulate the enzymatic activity of serine proteinases: the so-called “canonical” inhibitors and the serpins. The former are relatively small proteins (29–190 amino acid residues) that belong to numerous structural families (Bode and Huber, 1992). The latter are larger proteins (400–450 residues) that form a single family with highly conserved secondary structural elements (seven to nine  $\alpha$ -helices and three  $\beta$ -sheets) (Gils and Declerck, 1998; Silverman et al., 2001).

Canonical inhibitors and serpins have quite different reaction mechanisms. The former are tight binding reversible inhibitors with a short and rigid RCL that forms a “lock-and-key” complex with the substrate binding site of the proteinase. This Michaelis-type complex is stabilized by a large number of noncovalent bonds which account for the high enzyme-inhibitor binding energy (Bode and Huber, 1992). In contrast, serpins are irreversible enzyme inhibitors with a long and flexible reactive site loop of  $\sim 17$  amino acid residues of which eight residues (referred to as P<sub>5</sub> to P<sub>3</sub>) form the proteinase recognition site. They also form a Michaelis-type complex with their cognate proteinases, but this complex

represents the initial but not the final inhibitory complex. The latter is irreversible and SDS-stable as a result of the formation of a nonhydrolyzable ester linkage between O<sub>γ</sub> of the catalytic site serine residue of the proteinase and the carbonyl group of the P<sub>1</sub> residue of the inhibitor. This reaction is followed (Lawrence et al., 1995; Wilczynska et al., 1995) or preceded (Mellet and Bieth, 2000; Mellet et al., 2002) by translocation of the enzyme. In the final inhibitory complex the proteinase is firmly bound to the opposite pole of the serpin, the P<sub>15</sub>-P<sub>1</sub> part of the serpin's RCL inserts into  $\beta$ -sheet A to form the central strand of this sheet and the active site of the proteinase is distorted, thus preventing hydrolysis of the ester bond that links the enzyme to the inhibitor (Huntington et al., 2000). A number of proteinases are also able to cleave serpins at their RCL without undergoing inhibition. This substrate-like reaction inactivates the serpin, results in a loop-sheet insertion similar to that described above (Loebermann et al., 1984; Baumann et al., 1991), and considerably stabilizes the conformation of the protein (Bruch et al., 1988; Carrell et al., 1991). Thus, active serpins with an exposed RCL are metastable proteins, which, after limited proteolysis, are converted into a thermodynamically more stable state in which most part of the reactive site loop is deeply buried in the hydrophobic core of the protein. This dramatic conformational rearrangement is a key step of serpin's inhibitory mechanism.

Thermodynamics helps understanding of the mechanism of protein-protein interactions. The reversible binding of proteinases to canonical inhibitors is more amenable to thermodynamic description than the irreversible reaction of proteinases with serpins. The former is described by  $K_i$ , the equilibrium dissociation constant of the inhibitory complex from which  $\Delta G$ , the Gibbs free energy of binding may be calculated. Temperature-dependence of  $K_i$  (Menegatti et al.,

Submitted September 21, 2004, and accepted for publication December 22, 2004.

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**Abbreviations used:** PAI-1, recombinant wild-type plasminogen activator inhibitor-1; PAI-1-P<sub>14</sub>, recombinant plasminogen activator inhibitor-1 with a Thr→Pro mutation at position 333 (P<sub>14</sub>); RCL, reactive center loop; tPA, tissue-type plasminogen activator; PsE, *Pseudomonas aeruginosa* elastase.

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0006-3495/05/04/2848/07 \$2.00

doi: 10.1529/biophysj.104.053306

1984) or microcalorimetry (Freitas et al., 1999) give access to  $\Delta H$ , the enthalpy change of the reaction and hence to  $\Delta S$ , the entropy change. Of these three thermodynamic quantities, only  $\Delta H$  is measurable in the case of serpin/proteinase reactions because these reactions are irreversible, that is, thermodynamically complete (Boudier and Bieth, 2001).

Calorimetry shows that the reaction of  $\alpha_1$ -proteinase inhibitor and antithrombin with proteinases is highly exothermic with  $\Delta H$  values ranging from  $-20$  to  $-63$  kcal.mol $^{-1}$  (Boudier and Bieth, 2001). Since neither hydrolysis of the serpin's P<sub>1</sub>-P'<sub>1</sub> bond nor acylation of the proteinase's active site serine residue releases a significant amount of heat, we have hypothesized that the large  $\Delta H$  is mainly due to insertion of the N-terminal part of the serpin's RCL into  $\beta$ -sheet A (Boudier and Bieth, 2001).

Our study here uses PAI-1 in an attempt to verify this hypothesis. This serpin has the unique property of slowly self-inserting the N-terminal part of its RCL into its  $\beta$ -sheet A (Mottonen et al., 1992) with resulting loss of inhibitory activity (Heckman and Loskutoff, 1985) and increase of thermal stability (Munch et al., 1991). This so-called active to latent transition should, therefore, be accompanied by a significant production of heat if the above hypothesis is valid.

## EXPERIMENTAL PROCEDURES

### Materials

PAI-1 and PAI-1-T333P (PAI-1-P<sub>14</sub>) were expressed and purified as described previously (Gils et al., 1996). The two serpins were stored at  $-20^\circ\text{C}$  in 5 mM acetate buffer pH 5.0 containing 150 mM NaCl. Recombinant human tPA came from Boehringer Ingelheim (Brussels, Belgium). Porcine pancreatic trypsin was from Sigma and PsE was a gift from Dr K. Morihara, Japan. Active site titration of trypsin was performed with p-nitrophenyl-p'-guanidinobenzoate (Sigma-Aldrich, Saint Quentin Fallavier, France) (Chase and Shaw, 1969). The chromogenic substrate of tPA, spectrozyme tPA, came from American Diagnostica (Stamford, CT, USA). Acetyl-TVASSSTA, the octapeptide corresponding to the P<sub>14</sub>-P<sub>7</sub> sequence of the RCL of PAI-1, was synthesized for us by NeoMPS (Strasbourg, France).

### Calorimetric measurements

We used a VPITC microcalorimeter from Microcal Inc. (Northampton, MA, USA). Instrument control, data acquisition, and analysis were done with the VPViewer and Origin software provided by the manufacturer. The apparatus has a reference and a sample cell, the latter being equipped with a computer-controlled spinning syringe that ensures both reagent injection and permanent reaction medium mixing at a constant rate. Thermal equilibrium at the working temperature was reached in 10–20 min and reagent injection was done after 2–10 min of baseline acquisition. The differential electric power between the reference and the sample cell represents the raw experimental signal ( $\mu\text{cal.s}^{-1}$ ). The total heat resulting from an injection of titrant was calculated as the integral versus time of the experimental signal.

### Calorimetric investigation of the active to latent transition of PAI-1

The auto-inactivation of PAI-1 was investigated at  $37^\circ\text{C}$  by injecting 100  $\mu\text{l}$  (2.7 nmol) of PAI-1 stock solution in 5 mM acetate, 150 mM NaCl, pH 5.0

into the calorimeter sample cell filled with 1.4 ml of either 50 mM Hepes, 25 mM NaCl, pH 7.4 or 50 mM CHES buffer, 25 mM NaCl, pH 8.7. The effect of Triton X-100 was studied by mixing a 7  $\mu\text{l}$  aliquot of a buffered 2.5% detergent solution into the sample cell containing 4.6 nmol of PAI-1 in 50 mM Hepes, 150 mM NaCl, pH 7.4 and at  $37^\circ\text{C}$ . Control experiments with PAI-1-P<sub>14</sub> were done in 50 mM Hepes, 25 mM NaCl, pH 7.4 as with the wild-type serpin. The thermal power was monitored for 15–16 h.

### Calorimetric investigation of the reaction of PAI-1 with trypsin, tPA and PsE

These studies were done in 100 mM Hepes, 150 mM NaCl, pH 7.4 and  $25^\circ\text{C}$ . Titration of PAI-1 with trypsin was done by monitoring the thermal power generated by repeated injections of 6  $\mu\text{l}$  aliquots (in 10 s) of 64  $\mu\text{M}$  trypsin contained in the syringe to 1.4 ml of PAI-1 solution (1.53 nmol of active serpin) contained in the sample cell. Similarly, titration of tPA with PAI-1 was done by injecting 15  $\mu\text{l}$  aliquots (in 20 s) of 12.5  $\mu\text{M}$  PAI-1 to 1.4 ml of tPA solution (1.69 nmol of active enzyme).

The heat release resulting from the proteolysis of PAI-1 by PsE was recorded after injection of 20  $\mu\text{l}$  (in 30 s) of 1.8  $\mu\text{M}$  PsE into 1.4 ml of 0.67, 1.34, 2.68, 4.03, or 5.37  $\mu\text{M}$  PAI-1.

### Calorimetric investigation of the hydrolysis of peptide-bound PAI-1 by PsE

Acetyl-TVASSSTA was complexed with PAI-1 as already published (Kvassman et al., 1998). Complexing was checked using trypsin which is able to cleave the P<sub>1</sub>-P'<sub>1</sub> bond of peptide-bound PAI-1 without being inhibited. Cleavage was demonstrated electrophoretically (Kvassman et al., 1998). The heat release resulting from the proteolysis of peptide-bound PAI-1 was recorded after injection of 20  $\mu\text{l}$  (in 30 s) of 1.8  $\mu\text{M}$  PsE into 1.4 ml of 1.34 or 4.03  $\mu\text{M}$  peptide-bound PAI-1. Calorimetric recording was followed for 90 min.

### Inhibitory activity of PAI-1

Variable amounts of PAI-1 were mixed with 0.14  $\mu\text{M}$  tPA or 0.1  $\mu\text{M}$  trypsin in a total volume of 980  $\mu\text{l}$  of 100 mM Hepes, 150 mM NaCl, pH 7.4. After 10 min at  $25^\circ\text{C}$ , the residual tPA activity was determined by monitoring the absorbance at 405 nm after addition of 20  $\mu\text{l}$  of 25 mM Spectrozyme tPA. Free trypsin activity was measured after an incubation time of 1 h at  $25^\circ\text{C}$  as already described (Boudier and Bieth, 2001).

## RESULTS

### Calorimetric investigation of the active to latent transition of PAI-1

High ionic strength, low temperature, and low pH stabilize the active form of PAI-1 (Sancho et al., 1994). We have thus triggered the active to latent transition by transferring the serpin from a stabilizing environment (pH 5.0, 150 mM NaCl) to a medium that favors latency transition (pH 7.4,  $37^\circ\text{C}$ , low ionic strength). Curve A of Fig. 1 shows the calorimetric recording after addition of 2.7 nmol PAI-1 to the reaction cell filled with 50 mM Hepes, 25 mM NaCl, pH 7.4 at  $37^\circ\text{C}$ . A large peak of noise immediately followed the mixing of the two different buffers. After this short perturbation, the signal stabilized at the initial baseline level and remained flat during 16 h, a time sufficient to fully convert active PAI-1 into its

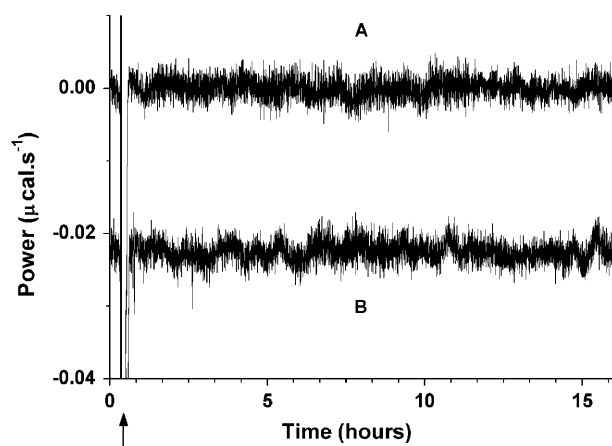


FIGURE 1 (A) Calorimetric data for the active to latent transition of PAI-1; 100  $\mu$ l (2.7 nmol) of stock solution of serpin was injected into 1.4 ml of 50 mM Hepes, 25mM NaCl, pH 7.4, 37°C, and the thermal power was recorded for 16 h. (B) Calorimetric data obtained from an identical experiment using the stable variant PAI-1-P<sub>14</sub> as a control of the stability of the calorimeter. The arrow indicates the time at which the samples of PAI-1 were injected into the calorimeter cell.

latent form ( $t_{1/2}$  for the active to latent conversion is  $\sim 2$  h). Measurement of the tPA inhibitory activity of the reaction medium after 16 h revealed that this activity was abolished to the extent of 95%, confirming that active PAI-1 had virtually fully converted into the latent form.

In further attempts to detect a possible heat release, we performed the same experiment using conditions that accelerate the active to latent transition. At pH 8.7 and at 37°C, PAI-1 has been shown to adopt its latent conformation two- to threefold faster than at pH 7.4 (Kvassman et al., 1995). Again no heat effect could be detected upon dilution of the same quantity of PAI-1 as above in the pH 7.4 buffer. On the other hand, 0.01% Triton X-100, a nonionic detergent, shortens the functional half-life of PAI-1 to 19 min at 37°C (Gils and Declerck, 1998). Again no heat production could be detected in the presence of this detergent. Due to the risk of both excessive disturbance of the signal and precipitation of PAI-1 in the low ionic strength buffer, we could not use larger quantities of the serpin.

To check the stability of the base line of the calorimeter, we run a control experiment using the PAI-1-P<sub>14</sub> variant which is not subject to structural alteration (Gils et al., 1996). The observed power versus time curve (curve B of Fig. 1) is quite superimposable to curve A, indicating that the lack of heat release observed for the active to latent transition of PAI-1 is not an artifact due to a drift of the base line of the calorimeter.

### Calorimetric investigation of the reaction of PAI-1 with tPA, trypsin and PsE

We wanted to see whether the lack of heat release is restricted to the self-insertion of the RCL into  $\beta$ -sheet A or

whether loop-sheet interactions resulting from the cleavage of the RCL that accompanies inhibition or substrate-like reactions are also energetically silent.

PAI-1 is the physiological inhibitor of both urokinase-type (u-PA) and tissue-type (tPA) plasminogen activators. Fig. 2 shows that the inhibition of tPA by PAI-1 is accompanied by a significant release of heat: each aliquot of PAI-1 causes a negative peak of thermal power up to saturation of the proteinase. Integration of the area under each peak yielded the quantity of heat released per aliquot of serpin. The inset of Fig. 2 depicts the heat release as a function of the molar ratio of PAI-1 to tPA and shows that the two partners tightly react with each other to form a stoichiometric complex. On the other hand, reaction of increasing amounts of PAI-1 with constant amounts of tPA and measurement of the residual enzyme activity with the tPA substrate, yielded a linear inhibition curve intercepting the x axis at a molar ratio of inhibitor to enzyme of one (data not shown). Linear titration curves characteristic of irreversible inhibition, are commonly observed with serpins (Beatty et al., 1980).

PAI-1 also inhibits other trypsin-like enzymes including bovine pancreatic trypsin (Kvassman et al., 1995). Preliminary experiments showed that PAI-1 inhibits porcine pancreatic trypsin at a much lower rate than tPA. This enzyme/inhibitor system was investigated as above except that aliquots of trypsin were mixed with the PAI-1 solution contained in the calorimetric cell (see also the experimental section). The interaction again resulted in a significant heat release and a calorimetric titration curve could again be constructed (Fig. 3). As can be seen, one mol of trypsin is necessary and sufficient to saturate one mol of PAI-1. Titration of trypsin with PAI-1 gave also a linear inhibition curve intercepting the abscissa at a molar ratio of inhibitor to enzyme of one, again confirming irreversible binding (data not shown).

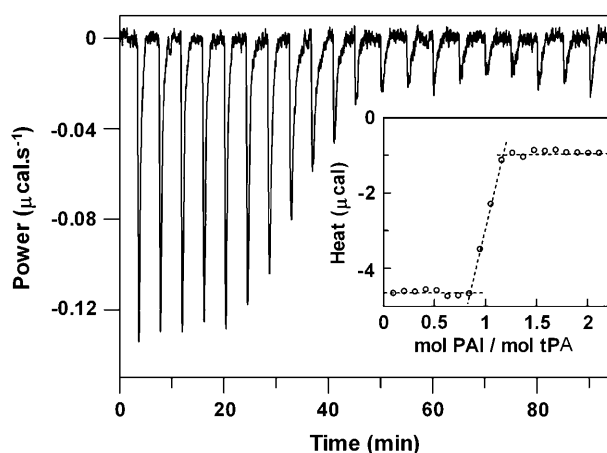


FIGURE 2 Thermal power generated during the inhibition of tPA by PAI-1 at pH 7.4 and 25°C. Aliquots of PAI-1 (0.18 nmol in 15  $\mu$ l) were successively added (in 20 s) to 1.4 ml buffer containing 1.69 nmol of active tPA while recording the thermal power. The inset shows the quantities of heat calculated from the area of each peak as a function of the molar ratio of PAI-1 to tPA.

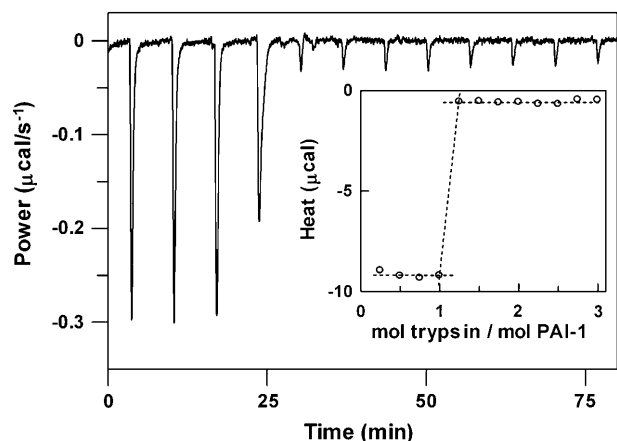


FIGURE 3 Thermal power generated during the inhibition of trypsin by PAI-1 at pH 7.4 and 25°C. Aliquots of trypsin (0.38 nmol in 6  $\mu$ l) were successively added (in 10 s) to 1.4 ml buffer containing 1.53 nmol of active PAI-1 while recording the thermal power. The inset shows the quantities of heat calculated from the area of each peak as a function of the molar ratio of trypsin to tPA.

The average quantities of heat produced during the calorimetric titration of tPA with PAI-1 (Fig. 2) and trypsin (Fig. 3) were calculated using the individual values from each experiment.  $\Delta H$  for the reaction of PAI-1 with tPA and trypsin was found to be  $-20.3 \pm 1.3$  and  $-22.5 \pm 1.4$  kcal.mol $^{-1}$ , respectively.

PAI-1 is inactivated by stromelysin-1, a matrix metalloproteinase, through peptide bond cleavage at its RCL (Lijnen et al., 2000). To study the heat release accompanying such a substrate-like reaction, we have used PsE, a bacterial metalloproteinase. In preliminary experiments PsE was found to inactivate PAI-1 in a few minutes when using an enzyme:inhibitor molar ratio of 1:10. Also, incubation of 7.5  $\mu$ M PAI-1 with 0.05–1.6  $\mu$ M PsE for 1 min at pH 7.4 and 25°C followed by SDS-polyacrylamide gradient gel electrophoresis showed that the active serpin was fully converted into a 40–41 kDa protein, indicating limited proteolysis (data not shown). Fig. 4 indicates that addition of 36 pmol PsE to varying amounts of PAI-1 generates negative peaks of thermal power. Further addition of enzyme caused no further calorimetric signal, indicating that the bulk of inhibitor was proteolyzed after injecting the initial enzyme sample (data not shown). The area under each peak was used to calculate the quantity of heat produced by each PAI-1 sample. The inset of Fig. 4 shows that this quantity is proportional to the quantity of serpin present in the reaction mixtures. The slope of the curve yielded  $\Delta H = -38.9 \pm 1.7$  kcal.mol $^{-1}$ .

### Calorimetric investigation of the hydrolysis of peptide-bound PAI-1 by PsE

To decide whether the aforementioned heat release is simply due to peptide bond cleavage at the RCL or to  $\beta$ -sheet

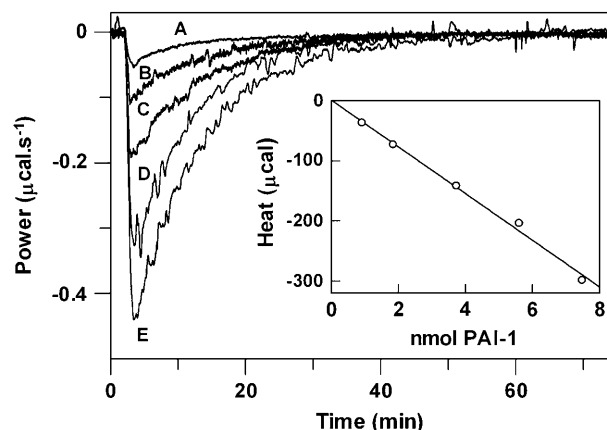


FIGURE 4 Thermal power generated during the PsE-catalyzed inactivation of PAI-1 at pH 7.4 and 24°C. The reaction was initiated by injecting 36 pmol PsE (20  $\mu$ l in 30 s) into 1.4 ml buffer containing varying amounts of PAI-1: 0.94 nmol (curve A), 1.88 nmol (curve B), 3.76 nmol (curve C), 5.64 nmol (curve D), and 7.52 nmol (curve E). The inset shows the quantity of heat as a function of the quantity of PAI-1 present in the reaction mixtures. The curve is theoretical and has been generated using the best estimate of  $\Delta H$  ( $-38.9$  kcal.mol $^{-1}$ ) calculated by linear regression analysis.

insertion resulting from this cleavage, we have run control experiments using peptide-bound PAI-1. Acetyl-TVASSSTA, the octapeptide corresponding to the P<sub>14</sub>-P<sub>7</sub> sequence of the RCL of PAI-1 was shown to insert into  $\beta$ -sheet A of the serpin and so prevents proteinase inhibition, that is,  $\beta$ -sheet insertion but not peptide bond hydrolysis at the RCL (Kvassman et al., 1995, 1998).

We have prepared peptide-bound PAI-1 under conditions where minimal amounts of latent PAI-1 accumulate (Kvassman et al., 1998). Electrophoresis showed that the binary complex was proteolyzed by both trypsin and PsE. Calorimetric measurements were done under conditions identical to those used for free PAI-1 (see Fig. 4), that is, 36 pmol PsE was reacted with either 1.88 or 5.64 nmol peptide-bound PAI-1. The calorimetric signal was rather low and the signal to noise ratio was poor (data not shown). The tentative value for  $\Delta H_{\text{proteolysis}}$  was  $-4$  kcal.mol $^{-1}$ , that is,  $\sim 10\%$  of the total enthalpy change observed with native PAI-1. In a previous article, we have shown that PsE cleaves peptide-bound  $\alpha_1$ -antitrypsin without significant heat release (Boudier and Bieth, 2001). There is no real discrepancy between the current data and the former data since we previously used a heat conduction microcalorimeter that is five- to 10-fold less sensitive than the apparatus used here. We may thus safely conclude that most of the energy released during the PsE-PAI-1 interaction results from insertion of part of the inhibitor's RCL into  $\beta$ -sheet A.

### DISCUSSION

PAI-1 reacts with proteinases according to the general serpin mechanism outlined in the introduction. For example, upon

reaction with plasminogen activator there is cleavage of its  $P_1$ - $P'_1$  peptide bond, covalent trapping of the proteinase and partial insertion of its RCL into the interior of the inhibitor (Lawrence et al., 1995; Wilczynska et al., 1995). Like other serpins, PAI-1 may be proteolyzed at its RCL without inhibition of the target proteinase (Aertgeerts et al., 1995; Shore et al., 1995). The proteolyzed inhibitor generated by this substrate reaction has a new  $\beta$ -strand (s4A) formed by insertion of the amino-terminal portion of the RCL into  $\beta$ -sheet A (Aertgeerts et al., 1995). A number of studies suggest that s4A is also present in the final inhibitory complex (Gils and Declerck, 1998) with the proteinase located at the opposite pole of the inhibitor as in the trypsin- $\alpha_1$ -antitrypsin complex (Huntington et al., 2000). The inhibition of tPA and trypsin by PAI-1 is therefore irreversible, that is, the association of the two partners is thermodynamically complete. Reversibility would imply i), extraction of s4A from  $\beta$ -sheet A; and ii), resynthesis of the  $P_1$ - $P'_1$  peptide bond, two highly energetically unfavorable steps which have never been observed in serpin reactions. The calorimetric and enzymatic titrations done here confirm the irreversible character of the binding. As a consequence,  $\Delta H$  is the only thermodynamic quantity that can be measured by calorimetry.

We have shown that both inhibition and substrate reactions of PAI-1 are strongly exothermic, a thermodynamic behavior already observed with  $\alpha_1$ -proteinase inhibitor and antithrombin III, two other members of the serpin family (Boudier and Bieth, 2001). The inhibition of both trypsin and tPA by PAI-1 is characterized by very similar  $\Delta H$  values ( $-20$  and  $-22$  kcal.mol $^{-1}$ ) although the two proteinases have quite different sizes ( $M_r = 24$  and  $67$  kDa, respectively) and are inhibited with quite different rates. In addition, these  $\Delta H$  values are close to those found for the inhibition of a variety of proteinases inactivated more or less rapidly by  $\alpha_1$ -antitrypsin and antithrombin ( $-20$  to  $-30$  kcal.mol $^{-1}$ ) (Boudier and Bieth, 2001). This strongly suggests that  $\Delta H$  solely originates from the dramatic structural rearrangement of the serpins accompanying inhibition. PAI-1 and the two other serpins share another feature: their substrate reaction is significantly more exothermic than their inhibition reaction. Since trypsin, elastase, and tPA are distorted in the final inhibitory complex (Huntington et al., 2000; Bousquet et al., 2003; Perron et al., 2003), it may be hypothesized that part of the energy released by the serpins' conformational change is utilized to distort the structure of the proteinase.

The classical thermodynamic hypothesis of protein folding holds that the three-dimensional structure of an active protein is the one in which the Gibbs free energy is the lowest. However, a number of proteins fold to a metastable state whose energy is higher than the classical free energy minimum (Baker and Agard, 1994). Serpins belong to the latter group: they fold in an active state which converts into an inactive state with significantly increased thermostability,

that is, lower free energy. Most of these inhibitors require proteolysis of the RCL (Bruch et al., 1988) or prolonged incubation under nonphysiological conditions (Wardell et al., 1997) to insert part of their RCL into  $\beta$ -sheet A and so attain their thermodynamic stability. In contrast, PAI-1 spontaneously undergoes this loop/sheet insertion under physiological conditions and within a reasonable period of time (Heckman and Loskutoff, 1985). We thought that heat release during the active to latent transition of this serpin would confirm our hypothesis that  $\Delta H$  observed for serpin: proteinase reactions (Boudier and Bieth, 2001) is indeed due to loop/sheet insertion. Results were contrary to expectation (Fig. 1). It should be stressed that the amount of PAI-1 used in the self-conversion experiment (Fig. 1) is 15- to 22-fold higher than that utilized in the proteinase-promoted heat-producing experiments (Figs. 2–4), indicating that the lack of thermal signal characterizing the active to latent conversion is not due to the use of too low amounts of inhibitor. Comparison of the three-dimensional structure of active, latent, and RCL-cleaved PAI-1 (Fig. 5) helps discussing this negative result. Latent and cleaved PAI-1 share a common topology of  $\beta$ -sheet A with an additional strand (s4A) originating from the N-terminal part of the RCL and displacement of helix F. However, these two forms considerably differ in the structure of the C-terminal part of the RCL: i), strand s1C, the C-terminal end of the RCL of active PAI-1, is present in the cleaved but not in the latent inhibitor; and ii), in latent but not in cleaved PAI-1 the remainder of the RCL exists in an extended conformation, which stretches along the surface of the protein. It may thus be hypothesized that during the latency transition of PAI-1, the energy released by the progressive insertion of s4A into sheet A is used to progressively extract s1C from  $\beta$ -sheet C and to let it adopt the elongated position shown in Fig. 5 B so that  $\Delta H = 0$  for the production of the latent inhibitor. In contrast, cleavage of the RCL during the inhibitor or the

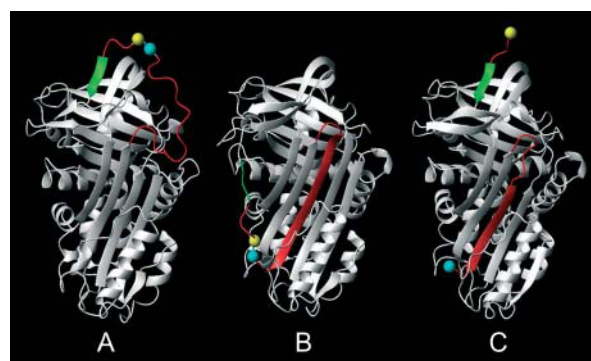


FIGURE 5 Comparison of the three-dimensional structures of (A) active (Nar et al., 2000), (B) latent (Mottonen et al., 1992), and (C) cleaved (Aertgeerts et al., 1995) PAI-1. The RCL is in red, the  $P_1$  and  $P'_1$  residues of the RCL are blue and yellow spheres, respectively, and strand s1C is in green. In latent PAI-1, s1C extracted from sheet C is shown in green as an extension of the RCL. Figure prepared with MOLMOL (Koradi et al., 1996).

substrate reaction of PAI-1 makes extraction of s1C unnecessary for loop/sheet insertion and explains why these reactions are highly exothermic. The fact that the latency transition is extremely slow as compared to the formation of cleaved inhibitor also argues in favor of an energy-demanding process.

Our calorimetric investigation shows that active and latent PAI-1, two conformers of the same protein, have identical total energies  $H$ , that is,  $\Delta H = 0$ . From the classical relationship  $\Delta G = \Delta H - T\Delta S$ , we may thus infer that  $\Delta G = -T\Delta S$ , that is, the active to latent transition of PAI-1 is entirely entropy-driven. Since the active to latent transition takes place spontaneously, it follows that  $\Delta G < 0$  and hence  $\Delta S > 0$ . This gain in entropy cannot be ascribed to a structural modification of PAI-1 during latency transition since the structure of latent PAI-1 is obviously less flexible than that of the active inhibitor (Fig. 5). It is more likely that entropy increases as a result of extrusion of water molecules from the surface of active PAI-1 when the RCL leaves its solvent-exposed position to insert in part into sheet A and to stick in part on the protein surface.

We thank Dr. K. Morihara for his generous gift of *Ps. aeruginosa* elastase. A.G. is a postdoctoral fellow of the Fund for Scientific Research-Flanders.

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